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PRINCIPAL INVESTIGATOR: Thomas Langan, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado
Health Science Center
Denver, Colorado 80262

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6. AUTHOR(S)
Thomas Langan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado – Health Science Center
Denver, Colorado 80262

E-MAIL:
thomas.langan@uchsc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

We have investigated two drugs which target cell cycle regulatory components as potential cancer chemotherapeutic agents for use in late stage breast cancer. The first, 5,6-dihydro-5-azacytidine (DHAC), a DNA methylation inhibitor, has been tested for its potential to reduce unregulated growth in cultured breast cancer cell lines which fail to express the cyclin-dependent kinase inhibitor protein p16 due to p16 gene methylation. DHAC was found to significantly affect the growth and cell cycle distribution of cultured T47-D breast cancer cells. However, this was not accompanied by detectable expression of p16 protein in these cells. We are currently employing a related methylation inhibitor, 5-aza-2'-deoxycytidine, in attempts to optimize conditions for promotion of detectable p16 expression, since this has the potential to produce greater reductions in cell growth as well as in tumorigenic properties. The second drug studied, bryostatin-1, promotes expression of another type of cyclin-dependent kinase inhibitor, p21. We have implemented a phase II clinical trial of bryostatin-1 in patients with advanced-stage breast cancer, and accrued five patients to this study. However, no positive responses have been observed to date. Bryostatin-1 does modulate the activity of protein kinase C, another target of its action, in treated patients as shown by measurements in peripheral blood mononuclear cells.

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Tina A. Longo 10/4/98

PI - Signature Date

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Introduction

During the past decade a very large increase in our knowledge of biological mechanisms regulating progression of cells through the cell division cycle has taken place. Together with the development of this knowledge, it has been shown by work in many laboratories that essentially all cancer cells have one or more defects in the components known to regulate cell cycle progression. For example, our recently completed studies of cell cycle regulatory defects in breast cancer cells, carried out with a grant from the Army Breast Cancer Research Program, showed that loss of expression of the cyclin-dependent kinase inhibitor p16, sometimes accompanied by overexpression of cyclin D1, is a common defect in breast cancer cells. These findings, plus the large amount of work carried out by others, presented a new potential target for cancer chemotherapy. Our proposal to exploit such targets for the chemotherapy of breast cancer is the basis for the current Clinical Translational Research Grant. We proposed to explore two drugs known or expected to cause changes in the expression of cell cycle regulatory components as potential chemotherapeutic agents in the treatment of late stage breast cancer. Bryostatin-1, shown by Kraft and coworkers to cause increases in the expression of the cyclin-dependent kinase inhibitor p21, was chosen as an agent to be tested in a phase II clinical trial. 5,6-dihydro-5-azacytidine, a DNA methylation inhibitor with less toxicity than the commonly studied 5-aza-2'-deoxycytidine, was chosen for pre-clinical studies directed towards eventually implementing a phase II clinical trial of that drug. DNA methylase inhibitors have been shown to increase the expression of p16 protein in cells where lack of expression is due to methylation of the p16 gene. The results of the initial year of the grant are reported here. These results indicate that some modifications in the approaches originally proposed may be desirable to best achieve the goals of the grant.

Body of Report

Materials and Methods

Breast cancer cell lines and tumor material

The breast cancer cell lines T47-D, were obtained from the University of Colorado Cancer Center Tissue Culture Core Facility. The T24 bladder cancer cell line and HCT-15 colon cancer cell line were obtained from the American Type Culture Collection. Breast cancer cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 0.1 mM non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin. T24 cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine, and HCT-15 cells were cultured in MEM supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 2 mM L-glutamine.

Antibodies

The anti-p16 was obtained from Oncogene. The horseradish peroxidase-conjugated secondary antibody was obtained from Bio-Rad.

Treatment of cells with demethylating agents

Cells were seeded onto 10 cm plates and allowed to grow to 30-40% confluence before the addition of the appropriate concentration of either 5-Aza-CdR or DHAC (drugs were diluted in medium specific for each cell line). Cells were incubated at 37°C for the desired period of time and passaged when necessary with fresh medium plus or minus drug as per the experimental specifications.

Protein extraction and western blot analysis

Cells were harvested, washed in PBS, and resuspended in Laemmli sample buffer (Laemmli., 1970). The extracts were then boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Approximately 100 ug of each protein extract were subjected to SDS/PAGE and transferred to nitrocellulose (Schleicher and Schuell) for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Fluorescent activated cell sorter (FACS) analysis

Cells were harvested and washed twice in PBS. A minimum of 3×10^3 cells were then resuspended in 0.5 ml Krishan's stain (Krishan, 1975) and subjected to cell cycle distribution analysis by the FACS core of the Cancer Center, University of Colorado Health Sciences Center.

Results

I. 5,6-Dihydro-5-azacytidine (DHAC) treatment of breast cancer cell lines

Previous studies in this laboratory demonstrated that the breast cancer cell line T47-D lacks detectable p16 protein expression as determined by western analysis. Examination of the genome of these cells by PCR techniques revealed that the p16 gene is present in a hypermethylated, inactive form, accounting for the lack of p16 expression. We therefore chose this cell line for an initial investigation of the ability of the DNA methylation inhibitor, 5,6-dihydro-5-azacytidine (DHAC) to restore p16 expression and cell cycle regulatory control in breast cancer cells.

We based our initial experimental conditions for this investigation on the work of Voytek et al. (1977), who had studied the effects of DHAC on the growth of cultured mouse leukemic cells. These investigators found that treatment with 10-20 uM of DHAC for 12-24 hours markedly reduces the growth rate of these cells, although no other parameters such as cell cycle distribution or expression of specific proteins was examined. Accordingly, we treated logarithmic phase cultures of the T47-D breast cancer cell lines with 0, 5 and 20 uM DHAC for 24 hours after which fresh medium (lacking DHAC) was added and the cells harvested 24 hours post treatment. The cells were analyzed both for cell cycle distribution analysis by FACS, and p16 expression analysis by western blot. We observed a significant increase in the G1 population and a significant decrease in the S-phase population of cells treated with both concentrations of DHAC, relative to untreated cells (Figure 1). However, western blot analysis of extracts isolated from both untreated and treated cells with an antibody to p16 indicated no induction of p16 protein expression in the DHAC-treated cells. We repeated the experiment as described above, this time using DHAC concentrations of 0, 20, 40 and 60 uM to treat the cells for 24 hours. Again, we observed an increase in the G1 population and a marked decrease in the S-phase population of cells treated at all three concentrations of drug (Figure 2), but no detectable induction of p16 expression by western blot analysis.

These data indicated that concentrations of 5- 60 uM DHAC resulted in growth inhibition in the T47-D cell line, confirming the early reports of growth inhibition carried out in mouse leukemic cells described by Voytek et al. (1977). However, we were unable to induce the expression of p16 protein expression expected on the basis of current knowledge of the mechanism of action of DHAC, and the basis for p16 suppression in cells containing methylated p16 DNA. Thus, we are currently in the process of determining the optimal DHAC concentration and exposure time necessary to induce concomitant demethylation of the p16 gene and growth arrest in breast cancer cell lines. As a starting point for these studies, we turned to the much more widely studied methylation inhibitor, 5'-aza-2'-deoxycytidine (5-Aza-Cdr), which has been directly shown to induce expression of p16 protein in several human tumor cell lines containing hypermentylated p16 gene sequences. The rationale was that the use of 5-Aza-CdR might

provide an opportunity to more quickly determine the optimal conditions for p16 induction in breast cancer cell lines and to serve as guidelines for further DHAC experimental treatment strategies.

II. Induction of p16 protein expression in human cancer cells following exposure to 5-Aza-CdR

Otterson et al. (1998) previously reported p16 induction in two lung cancer cell lines following a 4-24 hour exposure to 0.1, 0.3 or 1 uM 5-Aza-CdR and subsequent growth in drug-free medium for a lag period of at least 3-4 days. We therefore treated the T47-D cells with 0, 0.1, 0.3, or 1 uM 5-Aza-CdR for 24 hours after which fresh medium (lacking 5-Aza-CdR) was added. The cells were then harvested after 24 hours or 4 days. As expected based upon the lag period following drug treatment observed by Otterson et al., the cells harvested at 24 hours post drug-treatment showed no growth inhibition relative to untreated cells, nor induction of p16 protein expression. At 4 days post-drug treatment, the T47-D cells showed a paradoxical drug concentration-dependent decrease in the G1 population and an increase in G2/M accumulation (Figure 3). However, there was no concomitant induction of p16 protein expression in the T47-D cells. It may be that the change in cell cycle distribution profile exhibited by the T47-D cells is due to the effects of 5-Aza-CdR on the expression of other cellular genes. However, other explanations are possible, and we are pursuing the effects of 5-Aza-Cdr on cell cycle distribution and p16 expression in T47-D breast cancer cells further.

Owing to the lack of a detectable effect of 5-Aza-CdR on p16 expression in the T47-D breast cancer cell line using the conditions described above, we decided to optimize the experimental conditions using two cell lines that were previously reported to successfully undergo 5-Aza-CdR-induced transcription of p16. Bender et al. (1998) continuously treated the T24 bladder cancer and HCT-15 colon cancer cell lines, respectively, with 0.1 and 1.0 uM 5-Aza-CdR for 9 days and demonstrated demethylation and reexpression of the p16 gene. We obtained these two cell lines and treated logarithmic phase cellular cultures with 1.0 □M 5-Aza-CdR for 0, 24 hours, 5 and 8 days, respectively. Following treatment the cells were analyzed both for cell cycle distribution by FACS and p16 protein expression by western blot. Whereas there were no significant changes in cellular morphology of either of the two cell lines following the 24 hours exposure to 5-Aza-CdR we observed marked cellular enlargement and cytotoxicity following continuous exposure to drug for 5 and 8 days. These observations correlated with high levels of p16 protein induction in the cells harvested at the 5 and 8 day timepoints (Figure 5). These data indicated that greater than 24 hours of continuous 5-Aza-CdR exposure was required for demethylation and induction of p16 gene expression. Thus, it is highly probably that the lack of p16 induction in the T47-D breast cancer cell line described in the previous experiment was due to an insufficiently long exposure to 5-Aza-CdR. We did not observe the expected change in the cell cycle distribution profiles of the T24 and HCT-15 cell lines, but instead again saw a decease in the G1 and an increase in the G2/M population of T24 bladder cancer cells that had been observed in T47-D cells treated with 5-Aza-Cdr (Figure 4). We are currently in the process of treating the breast cancer cell lines with 5-Aza-CdR using the conditions optimized for the T24

and HCT-15 cell lines. In addition we are experimenting with a range of lower doses of 5-Aza-CdR and different exposure times to determine the optimal conditions required both for p16-induction and sustained expression.

III. Implementation a phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin 1 in stage IV breast cancer patients.

A protocol entitled "A phase II trial of bryostatin-1 for the treatment of stage IV breast cancer" was implemented and accrual of patients begun at the University of Colorado Cancer Center.

Goals of Protocol

1) To determine in a phase II study the clinical response of patients with stage IV breast cancer

2) To evaluate the efficacy of two different bryostatin-1 regimens 40 ug/m2/day every other week as a three-day continuos infusion versus 25 ug/m2/day weekly as a 24 hour continuous infusion.

3) To estimate the pharmacokinetic parameters of bryostatin-1 when given as an infusion.

4) To evaluate the ability of bryostatin-1 to regulate lymphocyte function.

5) To measure the effects of bryostatin-1 on protein kinase C activity.

Patient eligibility criteria include measurable metastatic breast cancer. Patients may not have had more than two prior chemo or hormonal therapies. Patients must be at least 18 years old and 2 weeks prior to radiotherapy. Patients must have a performance status of 0-2 ECOG. Patients must have a platelet count > 100,000 and a total neutrophil of greater than 2000.

Exclusions from this protocol include untreated brain metatasis and concomitant chemo or hormonal therapy. Staging includes PT, PTT, CBC, chem 18, EKG, CXR, CT scans and tumor measurements.

Upon completing evaluation the patients are randomized to receive either a 24 hour infusion of 25 ug/m2 every week for 8 weeks or a 72 hour infusion of 40 ug/m2/day every other week. After completing 8 weeks of therapy the patients are reevaluated by examination of positive studies.

Protocol Results to Date

Five patients have been entered on this protocol and their characteristics are described in Table 1. The average age of these patients was 44 years old. Two patients had had bone marrow transplantation and high dose chemotherapy prior to receiving bryostatin-1 therapy. Of the five patients four were randomized to receive a 24 hr. infusion every week for 8 weeks, while one was randomized to receive a 72 hour infusion every other week.

Two patients completed a complete 8 weeks of therapy and were reevaluated and found to have progressed. One patient received only 3 dose of therapy and became short of breath. Evaluation of the patient demonstrated that she had significant increase in pleural effusions. She was therefore removed from the study.

Two patients were not evaluable for response. One patient received a 72 hr. infusion of bryostatin-1 and had grade 4 muscle pains and dropped out of the study. A second patient developed a fever on bryostatin-1 that was thought to be secondary to dermatomyositis. Therapy of this breast cancer-related syndrome required high dose steroids and removal of the patient from this protocol.

The side effects of this therapy included muscle pains graded in intensity from 1-4. Patients complained of fever, headache, and tumor pain but none of these complaints lead to removal from the protocol.

IV. Evaluation of the effects of bryostatin-1 on PKC levels in peripheral blood mononuclear cells following bryostatin-1 infusion,

In culture bryostatin-1 addition to tumor cells modulates the activity of protein kinase C causing the association of protein kinase C with the membrane and a marked decrease in the level of cytosolic protein kinase C To evaluate the ability of bryostatin-1 to regulate the activity this enzyme in patients we have measured changes in the cytosolic levels of kinase C in macrophages during and after bryostatin-1 infusion.

At the times specified in Table 2 during and after the bryostatin-1 infusion, blood was drawn and granulocytes isolated. These cells were lysed by freeze thawing and the S-100 cytosol assayed for protein kinase C activity using histone H1 as a substrate . Histone H1 was phosphorylated in the presence of gamma 32P-ATP and the radioactivity incorporated into the histone measured. Protein kinase C is an enzyme that depends on phospholipids and calcium for its activity. Background phosphorylation of histone H1 was measured by removing the phospholipid from the reaction mixture and subtracted from the phospholipid containing mixture.

Two patients were studied with this methodology who received 24 hour infusion, although the remainder of the samples are still to be analyzed. As shown in Table 2, at 24 hours of treatment and at 5 min post infusion the activity of protein kinase C in the

cytosol dropped to 17 and 18 % of pretreatment values. In both patients, this PKC activity rapidly returned to pretreatment levels as shown at both 1 and 4 hours after the termination of the infusion.

V. Determination of changes in lymphocyte surface markers during bryostatin-1 chemotherapy.

Incubation of bryostatin-1 with monocytes is known to activate macrophage-like differentiation and the induction of cell surface adherence markers CD11a,b,c. As another marker of bryostatin's biologic effects in patients, we have measured the changes in these markers during and after the infusion of bryostatin-1. In one patient T cell markers were measured as a control. Blood was drawn from patients at appropriate time points and analyzed for surface markers with FACS analysis.

The T cell markers from patient SH002 when compared to the preinfusion values do not show any changes at 24, 48, 72 or 96 hrs after the infusion was begun (Table 3). This patient received a 24 hr infusion of bryostatin-1. To evaluate changes in the adhesion markers CD18 and CD11a,b,c the preinfusion binding was set at 100% and the changes in each patient during and after the infusion were noted. Both patient BW001 and SH002 received a 24 hr infusion while MS003 received a 72 hr treatment. As detailed in Table 4, in contrast to the other markers, there is signficant variability in these cell surfrace measurements both during and after the bryostatin-1 infusion period.

VI. Determination of pharmacokinetic parameters for bryostatin-1.

To estimate levels of bryostatin-1 in the blood of patients we have developed a bioassay that examine the ability of bryostatin-1 to displace radioactive phorbol ester from binding to rat brain membranes. This assay was carried out in two patients, BW001 and SH002, both of which received a 24 hr infusion. The displacement by the serum of the binding was compared to a standard curve of bryostatin-1 values to get molarity of bryostatin-1 in the serum. The levels in the serum are shown in Table 5. Since the assay is carried out at or near the levels of sensitivity, it was not felt that the values obtained (0-3nM) in patient SH002 were significant . However, a number of the values measured in patient BW001 (6 nM and 5 nM) of bryostatin-1 were significantly over background.

Key Research Accomplishments

- An investigation of the actions of DHAC on expression of p16 protein in breast cancer cell lines has been initiated.
- Optimal conditions for the induction of p16 expression by DNA methylation inhibitors have been explored using 5-Aza-Cdr, as well as cell lines in addition to breast cancer cell lines.
- A phase II clinical trial of bryostatin 1 in patients with stage IV breast cancer has been implemented.
- Infusion of bryostatin –1 in patients has been shown to modulate the activity of protein kinase C, producing reduced cytosolic levels of this enzyme after a 24 hour infusion, as indicated by measurements in peripheral blood mononuclear cells.

Reportable Outcomes

There have been no reportable outcomes to date.

Conclusions

Our studies of the effects of DHAC on the growth and expression of p16 protein in breast cancer cell lines have shown that this drug significantly affects growth and cell cycle distribution of the cells. However, these effects were not accompanied by a detectable increase in p16 protein. It is possible that increases in p16 occur which are below the level of detection of the antibody used in our western blot procedure, since earlier studies in our laboratory on p16 produced from vectors transfected into breast cancer cell lines have shown that very low levels of p16 expression can have significant effects on growth and tumorigenic properties. We are therefore utilizing 5-Aza-Cdr (a better studied but more toxic DNA methylation inhibitor) as well as other cancer cell lines in attempts to optimize conditions for promoting p 16 expression, since a higher level of expression may well result in a larger and more effective reductions in growth and tumorigenic properetes.

In our ongoing clinical trial of bryostatin-1 as a chemotherapeutic agent for the treatment of late stage breast cancer, no responses have been observed to date in these heavily pretreated patients. All evaluable patients relapsed within 1 cycle or 8 weeks of therapy. One patient had only 3 doses. The 72 hour infusion was given to only a single patient who experienced sufficient muscle pain to drop out of the study.

Previous experience with the 72 hour infusion by Dr.. Kraft has demonstrated that this infusion is not well tolerated by patients. Of the 4 patients treated in previous protocols all had to either be removed from the protocol or require a decrease in dosage. Although the National Cancer Institute has asked us to try this schedule, it is likely that this dose will not be tolerated.

Although only a few patients have been treated with bryostatin-1, they have been heavily pretreated and did not respond. Obviously to get a true idea of whether this agent is active in this disease untreated patients would be more useful. However, it has been difficult to accrue to this protocol and in fact if patients could be entered after more therapy the accrual rate might be increased.

It is likely that a trial of a standard chemotherapeutic agent such as taxol with or without bryostatin-1 for newly diagnosed patients might be the best approach to studying this agent. This cannot be attempted till the current protocol is complete.

Studies of protein kinase C levels, one expected target of bryostatin-1 action, have demonstrated that the biologic effects of bryostatin-1 on protein kinase C increase over the 24 hour infusion period. However, after the infusion is completed the bryostatin-1 modulation of this enzyme rapidly disappears. These results might suggest that longer infusions (i.e. 72 hrs) might modulate protein kinase C and thus effect tumor growth. This result is consistent with the increased muscle pains experienced by patients receiving this infusion.

Although not included in the original SOW, we have also studied a second indicator of bryostatin-1 action, induction of macrophage cell surface markers CD11a,b,c, employing T-cell markers as a control. The infusion of bryostatin-1 into a patient did not cause any change in T cell markers. However, there was significant variability in the macrophage adherence markers CD11a,b,c and CD18. CD11c values appear to increase after the infusion is completed. Further patient analyses will be necessary to achieve statistical significance in this observation.

Studies directed towards determination of pharmacokinetic parameters for bryostatin-1 showed that one patient, BW001, had significant elevations in the level of bryostatin-1 in the serum. These levels rapidly dropped when the bryostatin-1 infusion was stopped. The results of these measurements are similar to those obtained with protein kinase C in which termination of the infusion leads to rapid drops in not only the levels of bryostatin-1 but also the biologic effects. However, the number of measurements that were significantly above the background level of detection was too small to allow the calculation of half-life and other pharmacokinetic parameters. We are currently exploring more sensitive assay procedures based on HPLC and other techniques which will allow evaluation of pharmacokinetics.

Relationship to Statement of Work

Task 1. To implement a Phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin-1 in Stage IV breast cancer patients.

The clinical trial has been initiated as described in Section III of Results.

Task2. To evaluate the outcome of bryostatin –1 chemotherapy in terms of effects on tumor response as well as on expression of p21 cdk inhibitor protein and cdk activity in biopsied tumor tissue.

Evaluation of the outcome of bryostatin-1 treatment on the patients accrued to date has been carried out as described in Section III of Results. Patients accrued to date have not been suitable for the biopsy procedures needed to provide tissue for measurements of p21 expression and cdk activity.

Task 3. To evaluate the effects of bryostatin-1 on PKC levels in peripheral blood mononuclear cells following bryostatin-1 infusion, and to determine pharmacokinetic parameters for bryostatin-1.

Evaluation of the effects of bryostatin-1 on PKC levels in peripheral blood mononuclear cells has been carried out on two patients to date, as described in Section IV of Results. Determination of pharmacokinetic parameters using current methodology has met with difficulties, as described in Section VI of Results.

In addition to the tasks outlined in the original SOW, changes in lymphocyte surface Markers during bryostatin-1 chemotherapy have been evaluated, as described in Section V of Results.

Task 4. To determine the effects of DHAC on p16 levels and on growth and tumor formation by breast cancer cells.

Studies of the effects of DHAC on p16 expression in breast cancer cell lines have been Initiated as described in Section I of Results. In addition, studies of a related DNA methylation inhibitor, 5'-aza-2'-deoxycytidine, on p16 expression have been undertaken as described in Section II of Results.

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Appendix

Figures 1 to 5

Tables 1 to 5

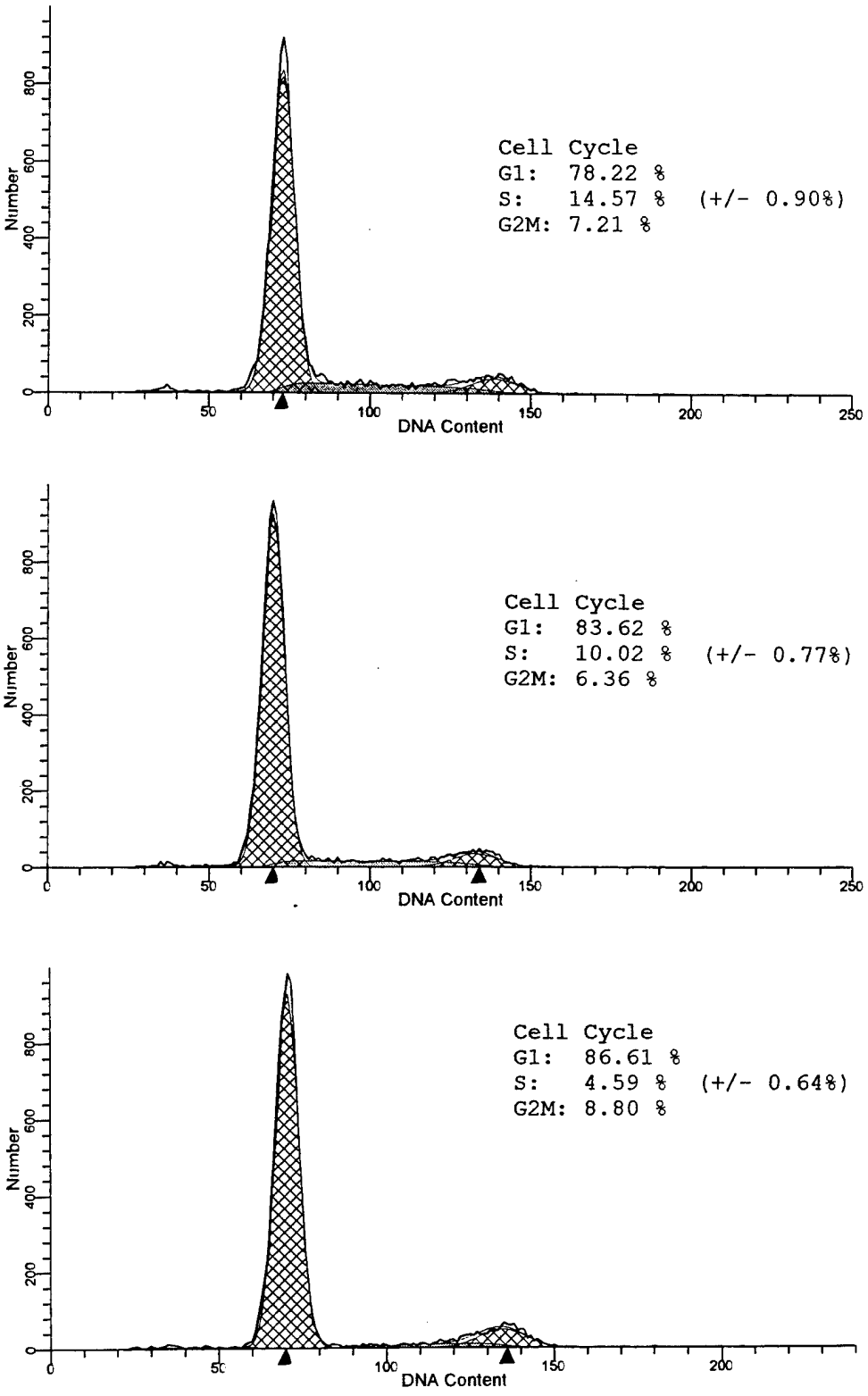

Figure 1. Logarithmic phase cultures of T47-D breast cancer cells were treated with 0 (top), 5 uM (center) and 20 uM (bottom) DHAC for 24 hours. Cells were harvested after further growth for 24 hours in DHAC-free medium and cell cycle distribution analyzed by FACS.

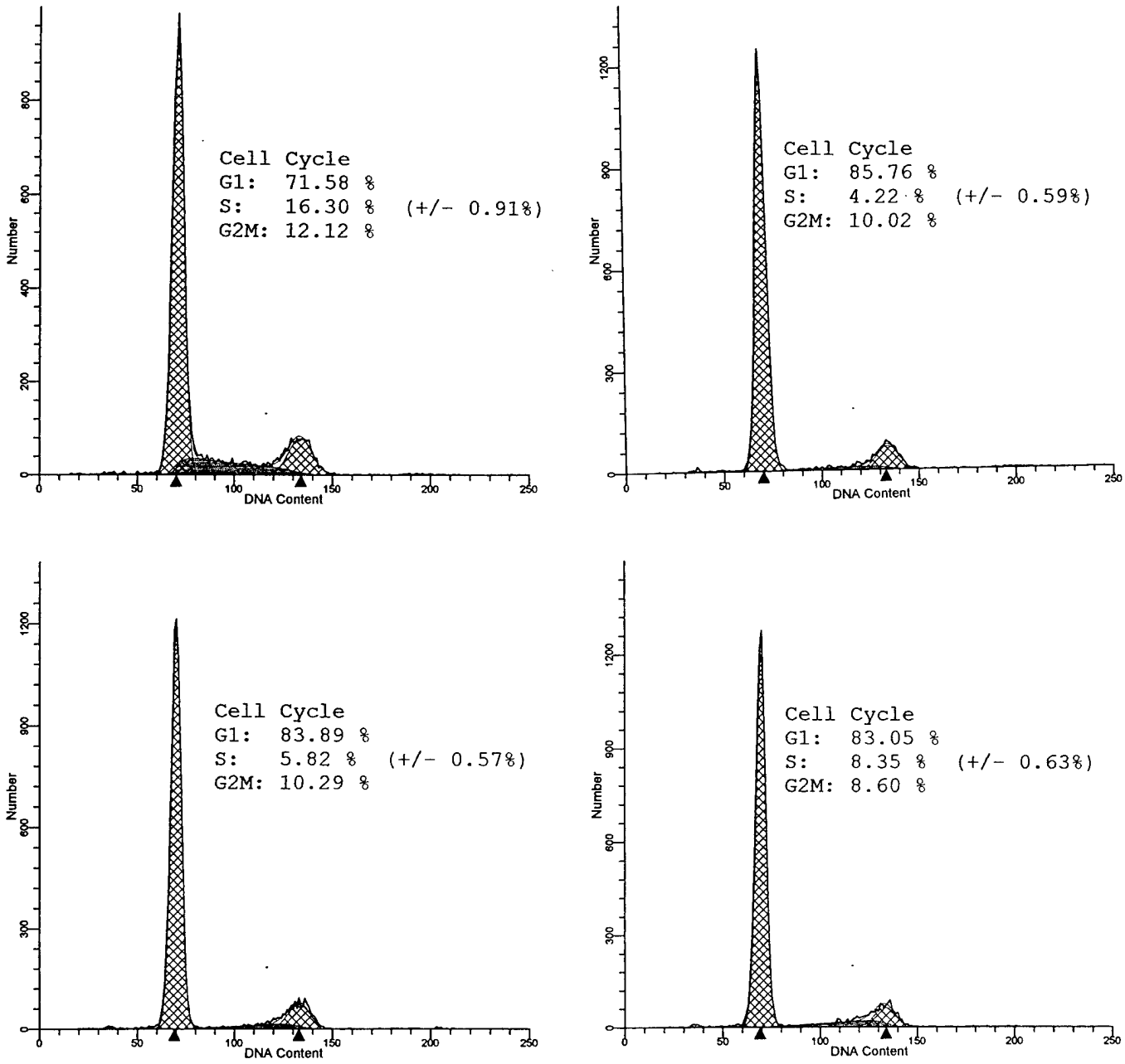

Figure 2. Logarithmic phase cultures of T47-D breast cancer cells were treated with 0 (top left), 20 uM (top right), 40 uM (bottom left) and 60 uM (bottom right) DHAC for 24 hours. Cells were harvested after further growth for 24 hours in DHAC-free medium and cell cycle distribution analyzed by FACS.

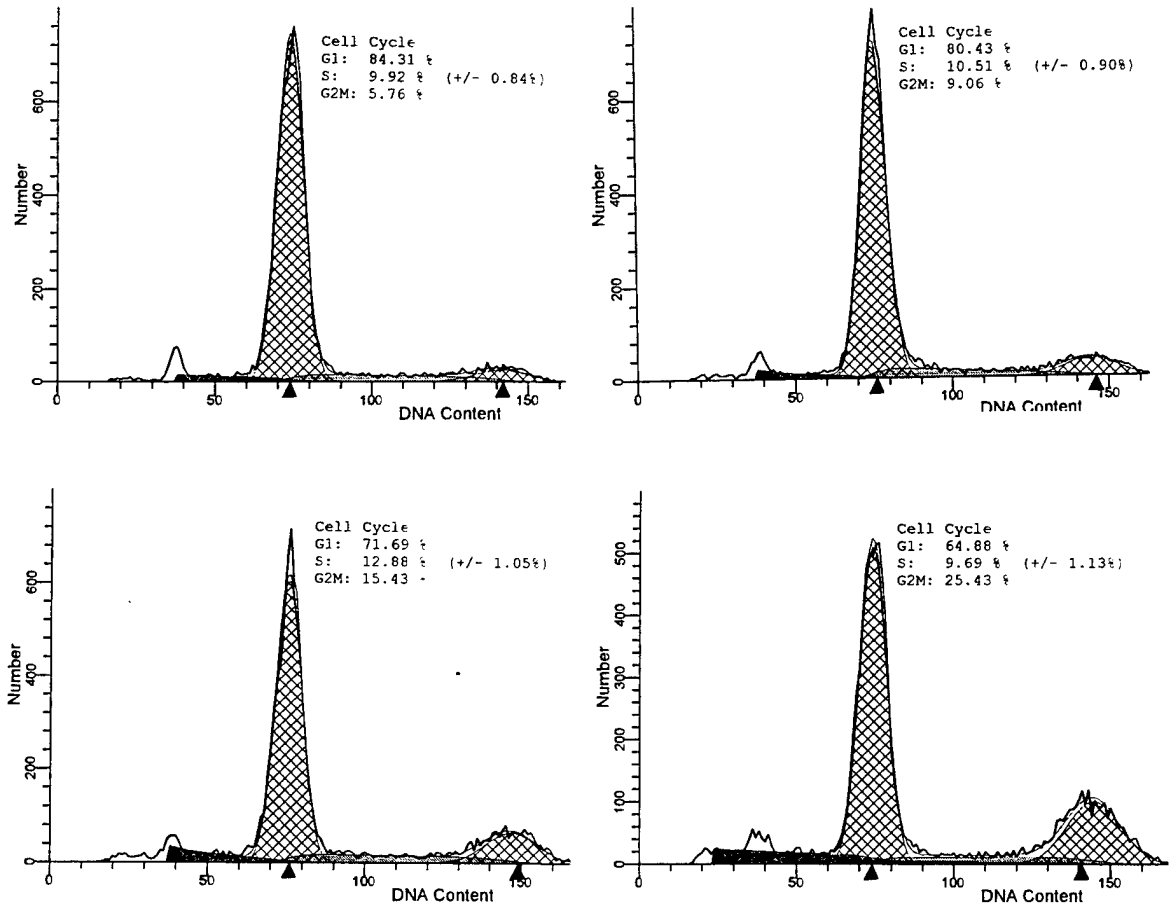

Figure 3. Logarithmic phase cultures of T47-D breast cancer cells were treated with 0 (top left), 0.1 uM (top right), 0.3 uM (bottom left) and 1.0 uM (bottom right) 5-Aza-Cdr for 24 hours. Cells were harvested after further growth for 96 hours in 5-Aza-Cdr-free medium and cell cycle distribution analyzed by FACS.

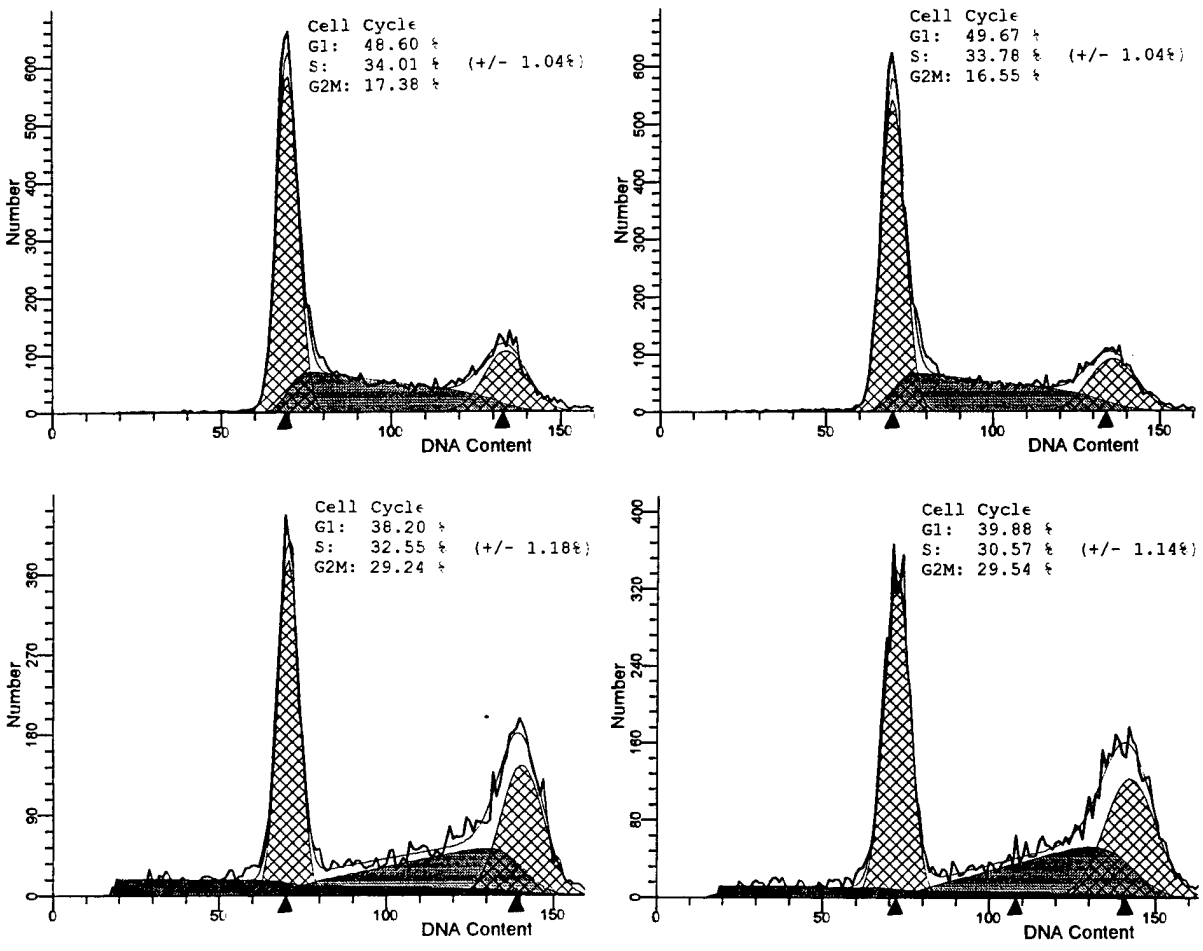

Figure 4. Logarithmic phase cultures of T24 bladder cancer cells were treated with 1.0 uM 5-Aza-Cdr for 0 (top left), 24 hours (top right), 5 days (bottom left) and 8 days (bottom right). Cells were harvested at the end of treatment and cell cycle distribution analyzed by FACS.

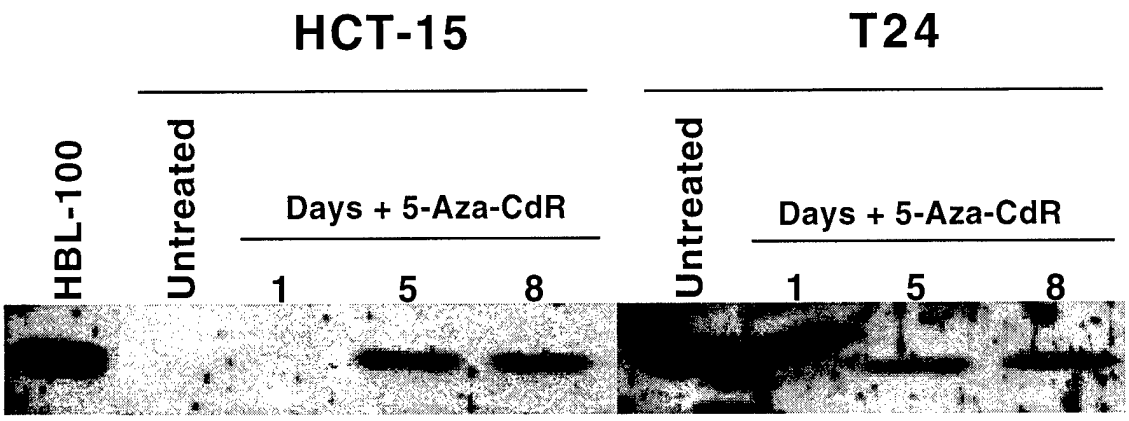

Figure 5. 5-Aza-CdR-induced p16 protein expression in T24 bladder cancer and HCT-15 colon cancer cell lines.
Logarithmic phase T24 and HCT-15 cells were treated with1 μM 5-Aza-CdR for 0, 1, 5 and 8 days at 37°C. Protein extracts isolated from both the untreated and treated cells were then separated by SDS/PAGE, transferred to a nitrocellulose filter and probed with an antibody to p16. The SV40-transformed HBL-100 cell line that overexpresses p16 protein was employed as a positive control. A significant induction of p16 protein expression was observed in extracts derived from cells that had been continuously treated with 5-Aza-CdR for 5 or 8 days.

Table 1: Characteristics of Patients Treated with Bryostatin

Age	Time of Infusion	No. of Doses	Outcome	Side Effects
54 years	24 hr	8 doses	Progressive	Headache, myalgia, decreased hemoglobin, increased alkaline phophatase
38 years	24 hr	8 doses	Progressive	Fever, Tumor Pain
46 years	72 hr	1 dose	Not Evaluable	+4 myalgia, muscle pain
41 years	24 hr	1 dose	Not Evaluable	Fever, + 2 myalgia dermatomyositis
39 years	24 hr	3 doses	Progressive	Mild muscle pain

TABLE 2: Protein Kinase Levels in Neutrophils pre- and post-Bryostatin Treatment

Patients	Time	+PL	-PL	Difference	Difference % Time
BW001	Pre	132276	22313	109963	100
	T+3hr	128960	19147	109813	100
	6hr	105240	16866	88374	80
	24hr	93004	23740	69264	63
	Post +5'	23626	4680	18946	17
	1hr	156916	31917	124999	113
	4hr	104602	14681	94921	86
	6hr	120563	17693	102870	93
	24hr	102680	18076	84304	76
SH002	Pre	100507	15675	84832	100
	T+3	113473	17274	96199	113
	6hr	110336	23265	87071	102
	24hr	70719	9491	61228	72
	Post +5'	17694	2370	15324	18
	1hr	74016	9737	64279	75
	4hr	99679	12137	87542	103
	6hr	73330	9378	63952	75
	24hr	94822	10491	80731	95

Values shown are counts per minute per microgram cytosolic protein.

TABLE 3: Lymphocyte Surface Markers

Patient	Marker	Preinfusion	24hr	48hr	72hr	96hr	120hr	144hr
BW 001	CD 11a	100%	90†	101	132	110		
SH 002			96	75	57	98		
*MS 003			64	56	95	92	84	85
BW 001	CD 11b	100%	-	-	-	-	-	-
SH 002			99	84	56	131		
*MS 003			23	23	57	59	100	96
BW 001	CD 11c	100%	142	234	198	132		
SH 002			112	131	78	161		
*MS 003			45	54	129	125	153	164
BW 001	CD 18	100%	92	102	178	113		
SH 002			92	60	55	100		
*MS 003			37	38	67	80	69	78

*MS 003 was on 72 hour protocol, BW 001 and SH 002 were a 24 hour infusion.

†All values expressed as percent of preinfusion values.

Table 4: Lymphocyte Surface Markers- Patient SH002

	CD 3+	CD 3+ CD 4+	CD 3+ CD 8+	CD 19+	CD 16+ CD 56+
Preinfusion	52.9+	22.0	31.8	27.7	15.7
24 hrs*	55.2	21.8	33.7	23.5	17.2
48 hrs	50.3	19.3	30.2	29.3	16.7
72 hrs	52.7	20.3	32.5	27.9	16.4
96 hrs	52.7	20.0	34.2	28.2	12.9

* end of infusion

† Absolute values average of triplicates

TABLE 5: Bryostatin Concentrations in Patient Sera (Nanomolar)

	Patient BW 001	Patient SH 002
Preinfusion	0	0
3 hr	6	-
6hr	2	-
24 hr	5	2.5
Post infusion 5'	3	-
15'	0	1.7
30'	-	3
1 hr	-	2.8
2 hr	-	2
4 hr	-	3
6 hr	-	1.7

"-" indicates below range of standard curve. Numerical values are in units of 10-9 M.